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Invited Review

Src protein tyrosine kinase family and acute inflammatory responses

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Okutani, Daisuke, Monika Lodyga, Bing Han, and Mingyao Liu. Src protein tyrosine kinase family and acute inflammatory responses. *Am J Physiol Lung Cell Mol Physiol* 291: L129–L141, 2006; doi:10.1152/ajplung.00261.2005.—Acute inflammatory responses are one of the major underlying mechanisms for tissue damage of multiple diseases, such as ischemia-reperfusion injury, sepsis, and acute lung injury. By use of cellular and molecular approaches and transgenic animals, Src protein tyrosine kinase (PTK) family members have been identified to be essential for the recruitment and activation of monocytes, macrophages, neutrophils, and other immune cells. Src PTKs also play a critical role in the regulation of vascular permeability and inflammatory responses in tissue cells. Importantly, animal studies have demonstrated that small chemical inhibitors for Src PTKs attenuate tissue injury and improve survival from a variety of pathological conditions related to acute inflammatory responses. Further investigation may lead to the clinical application of these inhibitors as drugs for ischemia-reperfusion injury (such as stroke and myocardial infarction), sepsis, acute lung injury, and multiple organ dysfunction syndrome.

inflammation; ischemia-reperfusion; sepsis; acute respiratory distress syndrome; multiple organ dysfunction syndrome; signal transduction; vascular permeability

SYSTEMIC INFLAMMATORY RESPONSE SYNDROME (SIRS) can be triggered by a number of factors such as sepsis, hemorrhagic shock, ischemia-reperfusion, severe burns, trauma, and major surgical procedures. Uncontrolled SIRS may lead to acute lung injury (ALI) or its severe form, acute respiratory distress syndrome (ARDS), and eventually lead to multiple organ dysfunction syndrome (MODS), which is often irreversible with mortality ranging from 60 to 98% (16). Many anti-inflammatory therapeutic strategies have been attempted to block one or more inflammatory mediators, but the clinical outcome of these studies is far from satisfactory. The search for new biomarkers (76) and therapeutic strategies (78) for ARDS and MODS has been a continuous effort for many research teams. At the cellular level, proinflammatory mediators activate a series of intracellular signaling pathways, most of which are regulated by protein phosphorylation, controlled by the balance of activities between protein kinases and phosphotases. Src protein tyrosine kinases (PTKs) are one of the most important families for intracellular signal transduction related to acute inflammatory responses (9, 84, 150). As the first proto-oncogene identified and the first protein demonstrated to possess intrinsic kinase activity, Src has been extensively studied in tumorigenesis. Chemical inhibitors directly or indirectly targeting Src PTKs have been developed as potential drugs for cancer therapeutics (71). However, most of these inhibitors in the clinical trials are for receptor tyrosine kinases, and the application of Src inhibitors is still at the preclinical stage (138). On the other hand, a number of animal studies have demonstrated that inhibition of Src PTKs with small chemical inhibitors is beneficial to prevent ischemia-reperfusion-induced injury in the brain and heart and to attenuate sepsis, ALI, and other organ damage. In this review article, we will briefly discuss the potential mechanisms of Src PTKs in acute inflammatory responses, focus on the in vivo studies, and then provide a perspective for the application of Src inhibitors as therapies for ARDS and MODS.

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BIOLOGY OF SRC PTK FAMILY

PTKs consist of two groups: receptor tyrosine kinases and nonreceptor tyrosine kinases; both have been considered as the primary mediators of intracellular signal transduction molecules in multicellular organisms (115). Tyrosine kinases are normally under tight control and have low basal activity; they are activated transiently in response to specific stimuli (132). Although receptor tyrosine kinases are important in mediation of ligand-specific responses, nonreceptor tyrosine kinases may have more broad functions and interact with receptor tyrosine kinases and other signaling mechanisms intracellularly.

Src PTK family is categorized into nonreceptor tyrosine kinases and consists of nine members (132) (Table 1). Src, Fyn, Yes, and Yrk are ubiquitously expressed, whereas Blk, Fgr, Hck, Lck, and Lyn are expressed in more restricted patterns (132). So far, Yrk has been detected only in the chicken (132). Several Src PTK family members, Blk, Fgr, Fyn, Hck, Lck, Lyn, and Yes, are critical in signaling pathways in hematopoietic lineages (84). For example, Lck and Fyn are expressed in T cells and are the first signaling molecules to be activated downstream of the T cell receptor (11). Expressions of Hck, Lyn, and Fgr are increased by multiple inflammatory stimuli, including lipopolysaccharide (LPS), in mature monocytes and macrophages (20, 154).

Src PTK members share a common architecture that underlies a common regulatory mechanism (141). The NH_2-terminal unique domain is required for membrane attachment and is

Table 1. *Expression of Src PTKs and specificities of Src PTK inhibitors (IC_{50}, nM)*

	Distribution	PP1	PP2	SU-6656	SKI-606	AZM 475271	M 475271	CGP 76030	CGP 77675
Src	ubiquitous	170	100	280	30	10	25	160	150
Fyn	ubiquitous	100	5	170					
Yes	ubiquitous			20		80	10		10
Yrk	ubiquitous								
Blk	G, Mo, Ma, B cells								
Fgr	G, Mo, Ma								
Hck	G, Mo, Ma		5						
Lck	T cells, B cells	5	4	688,000		30	200		290
Lyn	platelet, G, Mo, Ma, B cells	6		130					
Ref. no.		*	*	*	41, 42	148	152	56, 111	94, 110

*Data from: http://www.proteinkinase.de/html/tyrosine_kinase_inhibitors.html#PP2. G, granulocytes; Mo, monocytes; Ma, macrophages.

known as the Src homology (SH) 4 region. It is followed by the regulatory SH3 and SH2 domains, which are highly conserved and bind proline-rich and phosphotyrosyl regions, respectively. Through these interactions these domains participate in intra- and intermolecular regulation of kinase activity and determine the localization and substrate recognition of Src PTKs (141). Following the SH3 and SH2 domains are the catalytic domain and the COOH-terminal tail. The activity of Src PTK members is upregulated by phosphorylation of the tyrosine in the catalytic region (Tyr416 in Src) and negatively regulated by phosphorylation of the tyrosine in the COOH-terminal tail (Tyr527 in Src) (141).

Src PTK family members are activated in response to the stimulation of a variety of cell surface receptors (132), such as tyrosine kinase receptors, integrin receptors, and G protein-coupled receptors, and by cellular stress (48) (Figs. 1–3). Src PTKs can also regulate the functional activity of these receptors (132, 141). Src PTKs phosphorylate substrates in the cytosol or at the inner face of the plasma membrane (for example, Shc, Rho GTPase-activating protein p190, and transcription factor STAT3) (21); substrates at cell-matrix adhesions [cytoskeleton-associated proteins such as focal adhesion kinase (FAK), Cas, paxillin, ezrin, and cortactin] (18); and substrates at cell-cell adhesions (junctional proteins such as β-catenin, p120, and plakoglobin) (3). The complexity of the Src PTK family and their interactions with so many proteins related to signal transduction and cellular function place it into a very important position for the regulation of cell proliferation, differentiation, survival, metabolism, and other essential functions of the cells. One of the critical roles of Src PTKs is to regulate the inflammatory responses.

CELLULAR AND MOLECULAR MECHANISMS OF SRC PTKS IN INFLAMMATORY RESPONSES

A comprehensive review by Lowell (84) highlighted the signaling of Src PTKs in the immune cells. Here, we will use monocytes, macrophages, and neutrophils as examples to briefly illustrate how Src PTKs mediate the activation and function of immune cells. On the other hand, we will use endothelial and epithelial cells as examples to discuss the important role of Src PTKs in tissue cells in regulating vascular permeability and local inflammatory responses.

Src PTKs in Macrophages and Monocytes

Circulating monocytes and residential macrophages in the tissue are essential for inflammatory responses related to host defense as well as tissue damage. In response to stimulation by LPS and other pathogen products, these cells can produce multiple cytokines and chemokines, such as TNF-α, IL-1, and IL-6 (26). Hck, Fgr, and Lyn are the predominant Src PTKs in these cells (Table 1). It has been shown that LPS stimulation increased *hck* gene expression in human peripheral blood monocyte-derived macrophages (154). Furthermore, a combination of LPS and IFN-γ induced expression of Hck and Lyn in murine bone marrow-derived macrophages (20). These earlier studies demonstrated that the expression of Src family PTKs could be induced by inflammatory stimulations in monocytes and macrophages. More recent studies demonstrate that the Src PTK activities are also regulated during inflammatory responses.

CD14 is one of the important components found in the LPS receptor complex; it presents LPS to Toll-like receptor 4 (TLR4) and recruits multiple proteins related to signal transduction to the lipid rafts found on cytoplasmic membrane (136). It has been shown that Lyn could be coupled to CD14 in human monocytes (Fig. 1) and that LPS rapidly activated CD14-associated Lyn (124). Furthermore, LPS-induced activation of phosphatidylinositol 3 (PI3)-kinase involves its physical association with Lyn (52). Hck and Lyn kinase activity increased in monocytes after LPS stimulation, and blocking PTK activity with inhibitors reduced LPS-induced TNF-α production (13). Expression of a constitutively active mutant of Hck augmented LPS-induced TNF-α production in murine macrophages, whereas antisense oligonucleotides to Hck inhibited LPS-induced responses (32). Src PTK inhibitor PP1 also reduced ceramide-mediated inducible nitric oxide synthase expression and TNF-α production in murine macrophages (65). These studies demonstrated that these Src PTKs are involved in the activation of monocytes and macrophages. However, when Meng and Lowell (93) used macrophages isolated from $hck^{-/-}fgr^{-/-}lyn^{-/-}$ triple knockout mice, they found that the activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and JNK, as well as transcription factor NF-κB, was the same in normal and mutant macrophages. Moreover, nitrite production and cytokine (IL-1, IL-6, and TNF-α) secretion were also normal or even enhanced after LPS stimulation in macrophages derived from these $hck^{-/-}fgr^{-/-}lyn^{-/-}$ triple knockout mice, although the total protein phosphorylation level was greatly reduced (93). Given that the major signaling pathway for LPS in these cells is through TLR4, MyD88, TNF receptor-associated factor (TRAF) 6, IL-1 receptor-associated kinases, and other molecules (7),

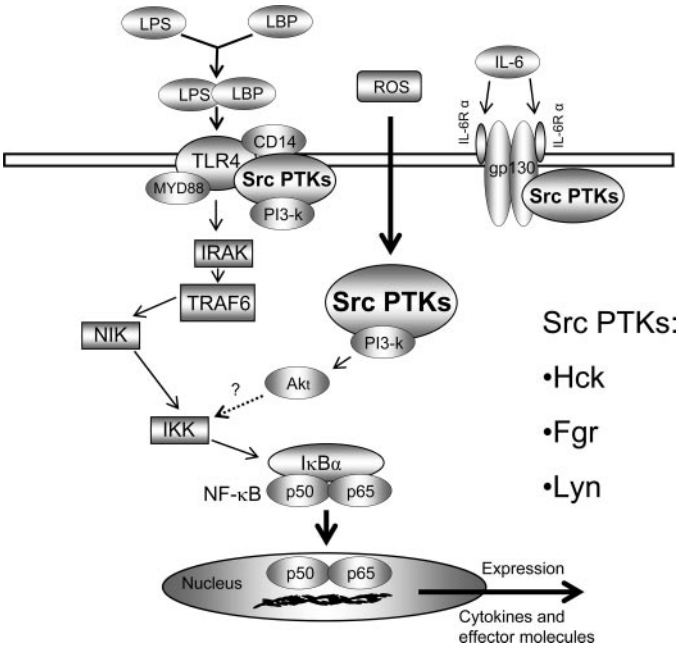

Fig. 1. Src protein tyrosine kinases (PTK)-related signaling in macrophages. Src PTKs (Hck, Lyn, Fgr) in macrophages are involved in signal transduction in many different ways. For example, LPS stimulation could recruit Src PTKs into the LPS receptor complex via CD14. The activation of Src PTKs may further recruit and activate phosphatidylinositol 3 (PI3)-kinase. Oxidative stress may activate Src PTKs, which enhance low-dose LPS-induced NF-κB activation through the PI3-kinase/Akt pathway. Src PTKs can also bind other receptors, such as IL-6 receptor (IL-6R) β-chain, gp130, in macrophages. LBP, LPS-binding protein; ROS, reactive oxygen species; TLR, Toll-like receptor; IRAK, IL-1 receptor-associated kinase; NIK, NF-κB-inducing kinase; TRAF, TNF receptor-associated factor.

results from these triple mutant studies indicate that Src PTK family members are important but not obligatory for LPS-induced signal transduction in monocytes or macrophages.

During acute inflammatory responses, multiple intracellular signal transduction pathways can be activated by different mechanisms. The cross talk between these pathways could be essential for activation of macrophages. For examples, Khadaroo et al. (63) showed that oxidant stress was able to augment macrophage responsiveness to subsequent low doses of LPS stimulation with earlier and enhanced NF-κB activation. Inhibition of Src family kinases either by PP2 or through cell transfection with Csk (Src COOH-terminal kinase that phosphorylates the inhibitory tyrosine residue of Src PTKs) prevented this effect. Interestingly, while Src inhibition was able to prevent the LPS-induced NF-κB translocation in oxidant-treated macrophages, it had no effect on NF-κB translocation caused by higher dose of LPS alone. Oxidative stress may divert LPS signaling along an alternative signaling pathway (63). Although oxidants altered the LPS-induced activation of p38, the latter did not appear to have a direct role in leading to oxidant-induced NF-κB translocation (64). In contrast, blocking the activation of PI3-kinase inhibited the oxidant-induced priming effect on LPS-induced NF-κB translocation (63). Together, these studies provide a novel potential mechanism whereby oxidants might prime macrophages for enhanced responsiveness to subsequent inflammatory stimuli (Fig. 1).

It should be pointed out that LPS is only one of the commonly used molecular tools for inflammatory studies; other pathogen products and proinflammatory mediators may also employ Src PTKs for their signal transduction. For example, it has been shown that Hck could be associated with the IL-6 receptor β-chain, gp130 (113). In addition to mediating the production of proinflammatory mediators, Src family PTKs are also involved in other cellular functions in macrophages. Migration and attachment of macrophages to the sites of inflammation and infection are critical for the macrophage function, which is mediated through integrins. Hck and Fgr have been found to be required for normal integrin-mediated signal transduction in murine macrophages (126), with Cbl, a proto-oncoprotein, as a potential downstream mediator for macrophage motility (23). The intracellular signal transduction pathways regulated by Src PTKs in monocytes and macrophages for inflammatory responses could be more complicated than what we know. Further investigations may lead to more detailed molecular mechanisms and potential targets for therapies.

Src PTKs and Neutrophil Recruitment and Activation

During inflammatory responses, neutrophils bind to endothelial cells and migrate into tissues leading to the release of reactive oxygen species (ROS) and granule constituents; this response requires adhesion of neutrophils to plasma membrane or extracellular matrix (ECM) proteins. The integrin receptors (principally β_2 and β_3) have been shown to be the major adhesive molecules that mediate neutrophil activation (101, 119, 153). Berton and coworkers (15) observed β_2-integrin-dependent protein tyrosine phosphorylation in human neutrophils, which was associated with activation of Src PTK family member, Fgr. They further demonstrated that the activation of both Fgr and Lyn correlated with neutrophil adhesion (147). Inhibition of endogenously produced reactive oxygen intermediates reduced the adhesion-stimulated activation of Fgr and Lyn in human neutrophils (146). On the basis of these and other observations, a molecular model system has been proposed that cytoskeletal proteins together with tyrosine kinases play an essential role in neutrophil adhesion and activation (146). TNF-α and/or tyrosine kinases may partially promote assembly of NADPH oxidase; full assembly of NADPH oxidase requires alteration of actin cytoskeleton structure mediated via cell adhesion. ROS generated by NADPH oxidase led to full activation of Fgr and Lyn and recruitment of other proteins into the signal transduction complex (146).

This model is further supported by studies with neutrophils from $hck^{-/-}fgr^{-/-}$ knockout mice (86). The respiratory burst was normal in $hck^{-/-}fgr^{-/-}$ neutrophils when stimulated by immune complex or phorbol 12-myristate 13-acetate, an activator of protein kinase C, but failed to undergo respiratory burst when plated on a surface coated with murine intercellular adhesion molecule 1 (ICAM-1). Direct cross-linking of the subunits of β_2- and β_3-integrins by surface-bound monoclonal antibody also failed to elicit O_2^- production. The impaired functional responses of $hck^{-/-}fgr^{-/-}$ neutrophils were caused by defective spreading and tight adhesion on either ECM protein- or monoclonal antibody-coated surfaces. Interestingly, the function of $hck^{-/-}$ or $fgr^{-/-}$ single mutant cells was similar to that of the wild-type neutrophils (86). These results suggest that adhesion and spreading on ECM through leukocyte β_2- and β_3-integrins are critical for neutrophil activation;

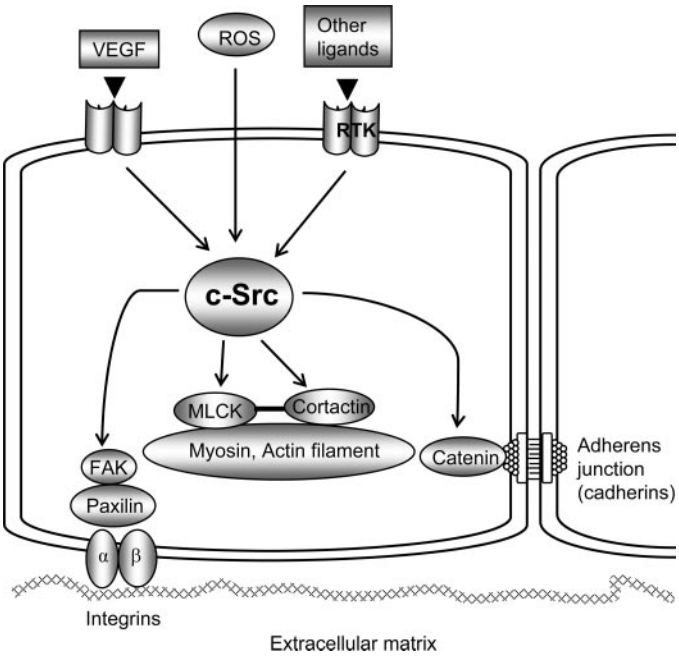

Fig. 2. Src PTK may play a central role in the regulation of endothelial integrity. Vascular permeability is regulated by contractile cytoskeleton, adherens junctions, and focal adhesions. Src-mediated tyrosine phosphorylation plays a critical role in all these three regulatory mechanisms. In endothelial cells, Src can be activated by ROS, VEGF, or other ligands through receptor tyrosine kinases (RTK). Phosphorylation of myosin light chain kinase (MLCK) by Src can lead to increased actin-myosin interaction and subsequent gap formation. Tyrosine phosphorylation of cadherin-catenin complex mediated by Src PTKs can promote the dissociation of junctional proteins from their cytoskeletal anchors. Focal adhesion kinase (FAK) can be phosphorylated directly by Src and closely associated with paxillin. Increases in tyrosine phosphorylation of FAK and paxillin may increase microvascular permeability.

it requires several Src PTK family members to ensure the proper function. In addition, it has been shown that adhesion-dependent degranulation of neutrophils also requires both Fgr and Hck (96). Bacterial tripeptide formyl-leucyl-methionyl-phenylalanine-induced p38 activation, and degranulation of primary and secondary granules was reduced in neutrophils isolated from the $hck^{-/-}fgr^{-/-}lyn^{-/-}$ mice (95). The requirement of several Src PTKs suggests that these cellular functions are very important; the redundancy of the Src PTKs could be important to maintain these functions. As will be discussed later, double or triple knockout animals have been used to determine the role of Src PTKs in vivo.

Src PTKs and Vascular Permeability

The microvascular barrier function is maintained in a semipermeable state by the balance between endothelial contractile cytoskeleton, adherens junctions, and focal adhesions. A disruption of the equilibrium results in endothelial barrier dysfunction and subsequent microvascular leakage (88). Src and its related signaling pathways are considered to be among the most critical mechanisms in regulating the structural changes occurring in the endothelium (98) (Fig. 2).

Endothelial contractile cytoskeleton. Actin and myosin are the major contractile components in the cytoskeleton. The actin-myosin interaction is mainly governed by the phosphorylation status of the regulatory myosin light chain (MLC) in vascular endothelial cells. Phosphorylation of MLC by myosin light chain kinase (MLCK) plays a critical role in the development and regulation of contractile forces within cells (60). Phosphorylated MLC led to the association of myosin with actin stress fibers to produce cell contraction (39, 151). Actin has been shown to undergo polymerization and redistribution to form stress fibers in association with intracellular clefts upon activation by neutrophils (55) or inflammatory agents (116, 120). MLCK-dependent MLC phosphorylation and cytoskeletal contraction affected the permeability of cultured endothelial cells and intact venular endothelium (39, 151).

Src has been recognized as an early-stage effector leading to MLCK-dependent MLC phosphorylation (134). Src can bind to MLCK and regulate its activity. Tyrosine phosphorylation catalyzed by Src contributes to the activation of MLCK under submaximal calcium concentrations, providing a mechanism that may tightly orchestrate cytoskeletal rearrangements critical for vascular barrier regulation (17). Mucha et al. (98) used a myosin phosphatase inhibitor, calyculin A, to elevate MLC phosphorylation and endothelial cell contraction, which activated Src kinase-dependent tyrosine phosphorylation of the focal adhesion proteins FAK and paxillin. These biochemical events led to rounding of endothelial cells and enlarged spaces between adjacent cells. The calyculin A-dependent monolayer hyperpermeability was reversed by Src family inhibitor, Herbimycin A.

Adherens junctions. Adherens junction serves as a major component in the regulation of paracellular permeability of the microvascular endothelium and inhibits the paracellular leakage of macromolecules. Vascular endothelial (VE)-cadherin is the primary component of adherens junctions connecting adjacent endothelial cells through a calcium-dependent homophilic binding of its extracellular domain, whereas its intracellular domain interacts with the actin cytoskeleton via a family of catenins, including α-catenin, β-catenin, γ-catenin, and p120 (12, 28). The cytoskeletal connection of VE-cadherin with catenin is important in maintaining junctional strength and paracellular permeability. Dissociation between VE-cadherin and catenin can cause endothelial barrier dysfunction (29, 143). β-Catenin plays an important role not only in structural linkage between VE-cadherin and other catenins but also in signal transduction of junction-cytoskeleton interactions (45, 54).

Tyrosine phosphorylation may promote the dissociation of junctional proteins from their cytoskeletal anchors (2, 143) and cause endothelial gap formation resulting in an increase in vascular permeability (6, 142). Polymorphonuclear leukocyte-induced hyperpermeability occurred with an increase in tyrosine phosphorylation of VE-cadherin and β-catenin (68); administration of PP1 attenuated neutrophil-induced hyperpermeability in both isolated coronary venules and cultured endothelial cells (73, 135). Vascular endothelial growth factor (VEGF) can disrupt VE-cadherin and β-catenin; Src activation after VEGF stimulation was responsible for tyrosine phosphorylation of VE-cadherin (74). Together, phosphorylation of both β-catenin and VE-cadherin by Src can be considered as an important signal capable of altering interactions between the junctional and cytoskeletal elements.

Focal adhesions. The attachment of endothelial cells to their ECM is mediated by focal adhesions composed of a family of

trans-membrane receptors, integrins (8). A group of intracellular proteins link integrins to the cytoskeleton (40). The cell-matrix interaction is dynamically controlled through assembly and disassembly of focal adhesions, in which integrins not only function as adhesion receptors but also transmit chemical signals and mechanical forces between the matrix and cytoskeleton (89, 121). The adhesive force generated by the interaction of integrin receptors with ECM at the focal adhesion complex regulates endothelial cell shape and serves to maintain the endothelial permeability (88). Blocking the integrin-ECM interaction increased endothelial permeability (144). Studies have shown that integrin engagement induced tyrosine phosphorylation of focal adhesion proteins found in focal adhesion complexes (150) and that Src was involved in integrin-induced tyrosine phosphorylation (31).

FAK and paxillin are Src substrates that are found in the focal adhesion complexes. The activity of FAK and paxillin is mainly regulated through phosphorylation, preferentially by the Src family PTKs (1, 109, 132). Association of Src with FAK may facilitate Src-mediated phosphorylation of tyrosine residues of FAK, some of which serve as binding sites for additional SH2-containing proteins (4, 114). Inflammatory mediators known to increase microvascular permeability can activate FAK through Src (4, 43). VEGF via Src induced the site-specific tyrosine phosphorylation of FAK, leading to the formation of a complex between FAK and $\alpha v\beta_5$-integrins, in both cultured endothelial cells and blood vessels. This complex was significantly reduced in endothelial cells from Src-deficient mice (31). The lack of VEGF-mediated vascular permeability from Src or β_5-knockout mice suggests that the VEGF-induced formation of the FAK/$\alpha v\beta_5$-complex via Src may be an important mechanism for coordinating growth factor-dependent integrin signaling in the regulation of vascular permeability (31).

Src PTKs and Epithelial Cells

During inflammatory responses, tissue cells, such as epithelial cells, fibroblasts, and smooth muscle cells also play an important role. For instance, there is increasing evidence to suggest that lung epithelial cells can actively participate in the inflammatory response (75, 122). These cells can produce a host of inflammatory mediators such as monocyte chemoattractant protein-1, IL-8, and IL-6 in response to inflammatory stimuli (75). Primary cultured rat pneumocytes produced TNF-α (90) and macrophage inflammatory protein-2 (145) in response to LPS stimulation. In addition to these well-defined cytokines, it has been recently shown that human lung epithelial cells produced long pentraxin PTX3, a newly discovered mediator of innate immunity and inflammatory responses, in response to TNF-α and IL-1β (49). Poynter and coworkers (108) demonstrated that selective inhibition of the NF-κB pathway in airway epithelial cells significantly reduced LPS-induced ALI, suggesting that lung epithelial cells may play a prominent role in orchestrating innate immunity and inflammatory responses in the lung.

Src PTKs are involved in the regulation of inflammatory responses in lung epithelial cells. *Pseudomonas aeruginosa*-induced mucin overproduction in epithelial cells required activation of NF-κB via an Src-dependent manner (72). TNF-α stimulation increased c-Src activation in human airway epithelial cells (57). In human lung epithelial cells, in addition to activating NF-κB-inducing kinase (NIK) via TRAF2, TNF-α could activate c-Src through protein kinase C. These two pathways converge at IKKβ; NIK may phosphorylate two serine residues, whereas Src PTKs may phosphorylate two tyrosines (Fig. 3). Activated IKKβ may go on to activate NF-κB via serine phosphorylation and degradation of IκB-α and finally to initiate expression of genes related to inflammatory responses, such as ICAM-1 (58) and cyclooxygenase-2 (59). Interestingly, redox-regulated c-Src activation could phosphorylate IκBα, which also led to activation of NF-κB (34) (Fig. 3).

The cellular and molecular studies have promoted investigations in animal models. Blocking Src PTK-related signal transduction could be a promising option to ameliorate tissue injuries related to excessive inflammatory responses. When Src PTK inhibitors are used in vivo, they may suppress inflammatory responses not only inhibiting the function of inflammatory cells, such as neutrophils, monocytes, and macrophages, but also reducing inflammatory responses of tissue cells, such as endothelial and epithelial cells.

SRC PTKS AND SEPSIS, INFECTION, AND ARDS

Tissue inflammation is the result of a complex set of interactions between various cell types and soluble factors, in

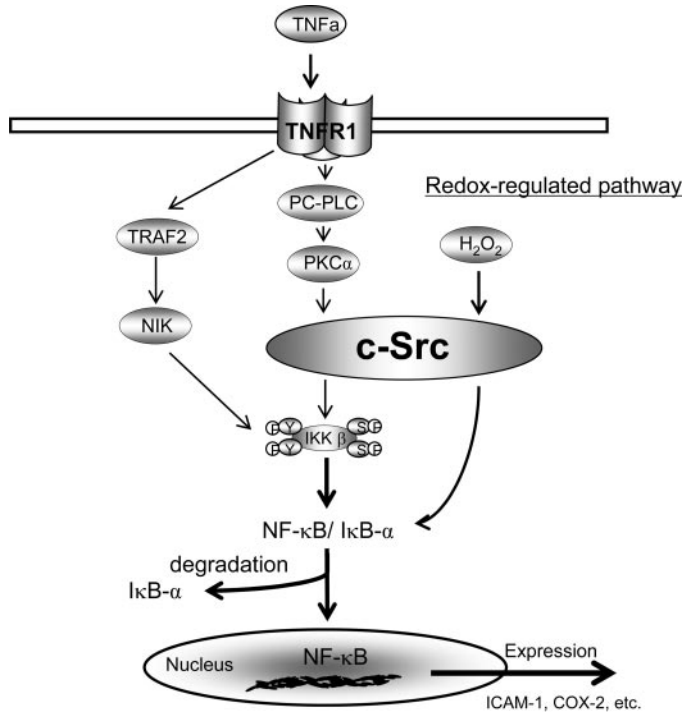

Fig. 3. Role of Src PTK in NF-κB activation in epithelial cells. The binding of TNF-α to TNF receptor-1 (TNFR1) activates NIK via TRAF2. In addition, TNF-α could activate c-Src through PKC. These 2 pathways converge at IKKβ; NIK may phosphorylate 2 serine residues, whereas Src PTKs may phosphorylate 2 tyrosines. Activated IKKβ may further activate NF-κB via serine phosphorylation and degradation of IκB-α. Translocation of NF-κB subunits into the nucleus lead to expression of genes related to inflammatory responses, such as ICAM-1 and cyclooxygenase (COX)-2. Redox-regulated c-Src activation could phosphorylate IκBα and lead to activation of NF-κB. PC-PLC, phosphatidylcholine-specific phospholipase C. P, phosphorylation; Y, tyrosine; S, serine.

response to traumatic, infectious, postischemic, toxic, or autoimmune injury (100). Inflammatory responses of the immune system to infection or irritation is one of the most important host defense mechanisms to protect the organism from severe illness (139). However, excessive inflammatory responses may lead to organ damage and dysfunction (100). As discussed above, adhesion of leukocytes to endothelial cells may lead to injury in microvascular beds (69). Increased inflammatory mediators may also target vascular endothelial cells leading to microcirculatory dysfunction (16). These inflammatory mediators can interact with immune cells and other tissue cells to elicit a series of intracellular signaling reactions. Activation of Src family PTKs is one of the most important mechanisms. Pharmacological inhibition of Src PTKs with small chemical compounds effectively protected animals from sepsis, viral infection, and other critical conditions (Table 2). Transgenic studies revealed roles of specific Src family members in different pathological settings (Table 3).

Pharmacological Inhibition of Src PTKs Reduced ALI

Many small chemical inhibitors for PTKs have been developed for cancer therapies (Table 1) (10), of which PP1, PP2, and SU-6656 have been used to investigate the roles of Src PTKs in conditions related to sepsis and ALI. PP1 and PP2 can bind to the ATP-binding site of the kinase (50, 80); both are potent and selective, especially for Hck, Lck, and Fyn (131). The activity of other nonreceptor tyrosine kinases, such as Janus kinase (JAK)-2 and Zap-70, is not directly affected by PP1 or PP2 (131). SU-6656 can inhibit several Src PTKs (Src, Fyn, Yes, and Lyn) with approximately equal potencies yet appears to be a poor inhibitor of Lck (19). Moreover, SU-6656 is selective for Src PTKs without inhibiting the PDGF receptor, whereas PDGF receptor catalytic activity can be reduced by PP1 and PP2 (19).

It has been shown that oxidant stress generated by hemorrhagic shock/resuscitation increased low-dose LPS-induced lung injury in rodents with increased translocation of NF-κB in alveolar macrophages as one of the proposed mechanisms (35). Khadaroo et al. (62) found that hemorrhagic shock/resuscitation caused a rise in Src PTK activity as shown by the increased phosphorylation of Hck in alveolar macrophages, which was prevented by treating animals during resuscitation with an antioxidant. Pretreatment of animals with PP2 reduced neutrophil sequestration in the lung and LPS-induced NF-κB translocation augmented by the shock/resuscitation in alveolar macrophages. Using cell culture as a model, this group further explored the molecular mechanisms of Src PTKs in priming the LPS-induced signal transduction in macrophages (50). Results from these in vitro and in vivo studies not only indicate that Src PTKs in macrophages may mediate the cross talk between different signal transduction pathways but also suggest the application of Src inhibitors as potential therapies for ALI.

Severgnini et al. (117) demonstrated that after intraperitoneal or intranasal LPS administration in mice, Src and JAK, another nonreceptor PTK, were rapidly activated in the lung together with the activation of STAT transcription factor. Systemic inhibition of these kinases using specific small molecule inhibitors for Src PTKs (either PP2 or SU-6656) significantly attenuated LPS-induced lung injury and capillary permeability and reduced LPS-dependent cytokine and chemokine levels in the lung and the serum (118). These inhibitors also reduced LPS-induced JAK activation and STAT3 phosphorylation in the lung tissue, suggesting that the JAK/STAT pathway is downstream from Src PTKs, because PP2 does not block JAK directly (131). When mice were given a lethal dose of LPS, these small molecule inhibitors significantly reduced mortality. Importantly, this protective effect was still evident even when the inhibitors were administered 6 h post-LPS challenge (118). These observations further suggest that Src PTKs participate in ALI and septic shock and that Src PTK inhibitors may serve as therapeutic agents for ARDS, sepsis, and other diseases related to SIRS.

Mechanical ventilation is an important life-supporting modality for critically ill patients. However, ventilator-induced lung injury has been identified as one of major detrimental components of ARDS and MODS (47, 48). Parker et al. (105) used an isolated-perfused rat lung model to demonstrate that protein tyrosine phosphatase inhibition increased the susceptibility of the lung to high-peak inflation pressure-induced lung injury, whereas tyrosine kinase inhibition attenuated the injury. Thus mechanical force-induced increase in protein tyrosine phosphorylation may contribute to ventilator-induced lung injury. Indeed, it has been shown that mechanical stretch activated Src PTKs in fetal rat lung cells (77, 83) and other cell types (46). By a microarray approach, it has been shown that mechanical stretch had additive effects on TNF-α-mediated gene expression in human lung epithelial cells (30). Stretch-induced Src activation may play a role in ventilator-induced lung injury on tissue cells, such as epithelial and endothelial cells (48).

Table 2. *Pharmacological inhibition of Src PTKs in acute inflammatory responses: evidence from in vivo studies*

Injury	Animal	Inhibitors (dose)	Main Findings	Ref. No.
ALI	Rat	PP2 (0.2 mg/kg iv)	Reduced alveolar macrophage priming and ALI	62
ALI	Mouse	PP2 (1 mg/kg ip), SU-6656 (8 mg/kg ip)	Decreased LPS-induced ALI and lethal-dose LPS-induced mortality	118
Ischemic brain injury	Rat	PP2 (1.5 mg/kg ip)	Reduced brain infarct size	67, 70
Brain injury	Rat	PP1 (2 mg/kg ip)	Reduced brain edema and mortality	67, 70
Spinal cord compression	Rat	PP1 (1.5 mg/kg ip)	Reduced contusional lesion, water content, and macrophage infiltration	5
Stroke	Mouse	PP1 (1.5 mg/kg ip)	Suppressed vascular permeability	106
Myocardial infarction	Rat	PP1 (5 mg/kg ip)	Reduced edema and tissue injury	140
	Mouse	PP1 (1.5 mg/kg ip)		

PTK, protein tyrosine kinase; ALI, acute lung injury; PP, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine.

Table 3. *Specific functions of Src PTKs in inflammation: evidence from transgenic studies*

	Functions	Ref. No.
$src^{-/-}$	VEGF-mediated vascular permeability	106, 140
$fyn^{-/-}$	Thymocytes/T cell signaling	125
	Natural killer cell development	38
	Fasting-induced thymic involution	102
$lck^{-/-}$	Involvement in Coxsackie virus B3-induced myocarditis and dilated cardiomyopathy	79
$lyn^{-/-}$	Multiple defects in immune system	53
	Hyperresponsiveness to antigen stimulation in B lymphocytes	24, 25
	Enhance Th2 response, severe asthma	14
hck (+)	Increased TNF-α production from macrophages	32
	Spontaneous pulmonary inflammation	33
$fyn^{-/-}/lyn^{-/-}$	Lupus-like kidney disease	149
$hck^{-/-}/fgr^{-/-}$	Impairment of natural immunity	87
	Defective adhesion-dependent neutrophil functions	86
	Resistance to endotoxic shock and reduced neutrophil migration	85
$Hck^{-/-}/Fgr^{-/-}/Lyn^{-/-}$	Important but not obligatory for LPS-induced signal transduction	93
	Impaired macrophage migration	92
	Reduced FcRγ receptor-mediated phagocytosis in macrophages	37

Src PTKs in Endotoxemia and Viral Infection: Evidence From Transgenic Animal Studies

It is difficult to determine on the basis of pharmacological studies which group of Src family members is involved in which particular disease process; the selectivity of these inhibitors to different Src PTKs has not been fully tested (Table 1). Genetic manipulation of genes related to Src family members has provided direct evidence of the roles of particular PTKs in different inflammatory responses (Table 3). For example, in $fyn^{-/-}$ mice, thymocyte and T cell signaling (125) as well as natural killer cell development (38) were altered. Although Lyn is not expressed in T cells, $lyn^{-/-}$ mice developed severe asthma with increased Th2 responses (14). Lupus-like kidney disease was developed in $fyn^{-/-}lyn^{-/-}$ mice (149). These Src family members are essential for allergic or autoimmune diseases mediated by lymphocytes. We will focus on Src family members related to infection, sepsis, and ARDS (Table 3).

Hck and Fgr are two Src PTK family members mainly found in granulocytes, including neutrophils, monocytes, and macrophage (Table 1). Lowell and Berton (85) examined inflammatory responses induced by LPS injection in $hck^{-/-}fgr^{-/-}$ mice. These animals showed marked resistance to the lethal effects of high-dose LPS despite the high levels of serum TNF-α and IL-1α, two of the most potent proinflammatory cytokines. These transgenic mice also showed reduced liver and kidney damage (85). Ernst et al. (33) used a "gain-of-function" approach to determine the physiological role of Hck, by generating Hck(F/F) "knock-in" mice that carry a targeted tyrosine (Y) to phenylalanine (F) substitution of the COOH-terminal negative regulatory Y(499)-residue in the Hck protein. These mice spontaneously acquired a lung pathology characterized by extensive eosinophilic and mononuclear cell infiltration within the lung parenchyma, alveolar air spaces, and around blood vessels, as well as marked epithelial mucus metaplasia in conducting airways. When challenged transnasally with LPS, these mice displayed excessive release of matrix metalloproteinases and TNF-α; they were also highly sensitive to endotoxemia after systemic administration of LPS (33). These studies suggest that Hck and Fgr tyrosine kinase are important in inflammation-dependent tissue injury in vivo. As these two Src PTK members are mainly expressed in granulocytes, these studies suggest the role of these inflammatory cells in the endotoxemia.

Lck is a member of Src PTK family primarily expressed in lymphocytes (Table 1). Infection of wild-type mice with human pathogenic Coxsackie virus B3 (CVB3) caused acute and severe myocarditis, meningitis, hepatitis, pancreatitis, and dilated cardiomyopathy, whereas $lck^{-/-}$ mice were completely protected from virus-induced acute pathogenicity and chronic heart disease (79). These effects appeared to be mediated through ERK1/2 as downstream signals of Lck (104). The activation of lymphocytes and consequently excessive inflammatory responses are the major cause of death induced by CVB3; Lck and related signals play an essential role that controls the replication and pathogenicity of this virus.

SRC PTKS AND ISCHEMIA-REPERFUSION INJURY

The role of Src PTKs in ischemia-reperfusion injury is another good example to elucidate the importance of this family of PTKs in acute inflammatory responses. Activation of Src PTKs has been observed in multiple animal models from different organ systems. Blocking Src PTKs could attenuate tissue injury by reducing the increased vascular permeability (Table 2).

Ischemia-Reperfusion Activates Src PTKs in the Brain and Neural Tissues

Korematsu et al. (66) reported that occlusion of rat middle cerebral artery for 1 h led to a significant increase in protein tyrosine phosphorylation of the microglia in the insulted cerebral cortex 3 h postreperfusion, as analyzed with immunohistochemistry. The positively stained cells also demonstrated morphological changes, with shortened and thickened processes, enlarged cell bodies, and ameboid features. Increased protein tyrosine phosphorylation was also found in rat retina after ischemia-reperfusion injury, together with increased VEGF levels in the damaged tissues, and increased tyrosine phosphorylation of phospholipase C-γ, Shc, and MAP kinase, potential substrates of Src PTKs (51). Using a transient (15 min) bilateral carotid artery occlusion in gerbils, Pei et al. (107) demonstrated that total PTK as well as Src activity was increased significantly after ischemia-reperfusion. *N*-methyl-D-

aspartate (NMDA) receptor and L-type voltage-gated calcium channels appear to be involved in Src activation after ischemia-reperfusion; antagonists for NMDA receptor (82) or for L-type voltage-gated calcium channels (81) prevented ischemia-reperfusion-induced Src activation. One of the NMDA receptor subunits, NR2A, was found to form a complex with Src in the damaged brain tissue (82).

Systemic administration of Src inhibitor PP1, given up to 6 h following stroke, suppressed vascular permeability and protected mice from ischemia-induced brain damage. This was associated with reduced edema, improved cerebral perfusion, and decreased infarct volumes 24 h after injury, as measured by magnetic resonance imaging and histological analysis (106). PP1 also reduced secondary damage after spinal cord compression in rats; mRNA expression of proinflammatory cytokines, TNF and IL-1β, was also reduced in PP1-treated animals (5). Similar protective effects of Src inhibitors, either PP1 or PP2, have been shown by others with different models of brain injuries (67, 70). VEGF is a mitogen and survival factor for endothelial cell, as well as a potent mediator of vascular permeability (99). Mice lacking c-Src were resistant to VEGF-induced vascular permeability and showed decreased infarct volumes after stroke, whereas mice deficient in c-fyn, another Src family member, showed similar pathological changes as that of wild-type mice (106). These studies suggests that Src represents a key intermediate and novel therapeutic target in the pathophysiology of cerebral ischemia (106).

The underlying mechanisms of the clinical conditions represented by these ischemia-reperfusion animal models are complicated, each with unique features and clinical rationales. A common noticeable feature of these models is tissue damage and inflammatory responses. Although detailed information about particular Src PTK members is not clear for every model system, the protective effects of Src inhibitors observed suggest that the activation of Src PTK could be a shared mechanism and potential target for therapies.

Effects of Src PTK in Ischemia-Reperfusion Injury of Other Organ Systems

Activation of Src PTKs is also involved in ischemia-reperfusion injury in other organs. In perfused guinea pig hearts, ischemia alone stimulated activation of multiple signal transduction proteins, including p90 ribosomal S6 kinase, Src, and big MAP kinase-1 (BMK1). Src inhibitor PP2 suppressed ischemia-mediated BMK1 activation. However, the activities of both Src and BMK1 were markedly attenuated after reperfusion (129). Therefore, the role of Src activation in myocardial ischemia-reperfusion injury is unclear (9). Recently, Weis and coworkers (140) demonstrated that ischemia resulting from myocardial infarction led to increased vascular permeability and tissue injury throughout the ventricle. In $Src^{-/-}$ mice, or using PP1 to block Src activation, preserved endothelial cell barrier function, suppressed vascular permeability and infarct volume, long-term improvement in cardiac function, and survival after myocardial infarction were found.

The Src activity in a rat renal ischemia-reperfusion injury model was examined using a monoclonal antibody specific for the active form of Src kinases; increased active Src expression was found in the injured rat kidney 6 h after reperfusion, with peak activation at 12 h that was further confirmed by in vitro kinase assay (130).

Ischemia and ischemia-reperfusion represent many different clinical situations. For example, in the heart, preconditioning, transient ischemia-reperfusion injury, and permanent ischemia lead to different types of tissue injuries. On the basis of cellular and molecular studies, the Src PTK family appears to be implicated in all of these conditions but possibly through different mechanisms. The objective of this article is to use these models as examples to promote further investigation on Src PTKs in these pathological processes. The usage of Src inhibitors under different clinical condition merits further studies.

Src Activity During Lung Transplantation

Ischemia-reperfusion is an unavoidable step in solid organ transplantation. In the lung, ischemia-reperfusion-induced acute injury of transplanted grafts has been a subject of an extensive review (27); however, the intracellular signal transduction events are largely unknown. Keshavjee et al. (61) examined protein tyrosine phosphorylation status using samples collected during human lung transplantation. After the hypothermic preservation, the donor lung was implanted at room temperature. The total protein tyrosine phosphorylation was significantly increased compared with the levels after cold preservation; interestingly, protein phosphorylation was then significantly decreased during the first 2 h of graft reperfusion. The activity of Src PTKs and Src protein levels were reduced during graft reperfusion. They also examined these changes in a rat lung transplantation model with similar findings (112). These observations are intriguing, because it is opposite from what has been described above, data from the brain, heart, and kidney. This discrepancy might be due to the difference of pulmonary circulation vs. systemic circulation. However, most likely this represents a unique feature of organ transplantation. In other clinical settings, the organs experience lack of blood and oxygen at body temperature for a short period and then are reperfused by blood from the same individual. In transplantation settings, organs are usually preserved at low temperature for relatively longer periods, followed by reperfusion with warm blood of the recipient. Especially for lung preservation, the donor lung is inflated with oxygen for preservation; the lung tissues and cells are not deprived of oxygen. The cellular and molecular mechanisms of this particular type of ischemia-reperfusion could be unique. The hypoxia-reoxygenation condition commonly used at the cellular levels to determine the mechanisms of ischemia-reperfusion is not applicable for the investigation of lung transplantation. A cell culture model, with high concentration oxygen, low temperature, and incubation with preservation solution, has been used to simulate the process of lung preservation (22). Therefore, it is important to study the role of Src PTKs in pulmonary ischemia-reperfusion at body temperature, an issue related to pulmonary embolism and other clinical settings. It is also important to determine the role of Src PTKs in other organ transplantation settings. These comparative studies will help us understand the pathophysiological process of ALI associated with different types of ischemia-reperfusion conditions and will also help establish the importance of intracellular signal transduction events associated with organ transplantation. It will be interesting to see that

we might need to use protein tyrosine phosphatase inhibitors to ameliorate ischemia-reperfusion injury in transplantation settings.

PERSPECTIVE: SRC INHIBITION AS THERAPEUTICS FOR ACUTE INFLAMMATORY RESPONSES

As reviewed in this article, Src inhibitors have shown a great potential as therapeutic agents for diseases related to acute inflammatory responses, such as ischemia-reperfusion injury, sepsis, ARDS, and perhaps MODS. The transgenic studies indicate that Src itself may be crucial for the regulation of vascular permeability (106, 140), whereas Hck, Fgr, and Lyn may control the recruitment and activation of leukocytes (85–87, 92, 93). Blocking these Src PTK family members may be essential for those therapeutic effects observed in the pharmacological studies.

Recently, inhibition of PTK has become effective therapies for cancers; however, almost all clinical trials related to PTKs are using inhibitors for receptor tyrosine kinases (103, 133). Src inhibitors have only entered at the preclinical trial stage, despite rather clear understanding of the roles of Src PTKs in cell proliferation, differentiation, survival, and other functions (138). Diseases related to acute inflammatory responses could be an attractive option for the clinical application of these small molecule inhibitors. Compared with the cancer therapy, which requires a life-time administration of medication, ischemia-reperfusion, sepsis, ARDS, and MODS are acute. Patients are in critical conditions for days or weeks. If the therapy is effective, these Src inhibitors will be administrated only for a short period. Considering the importance of Src PTKs in the regulation of normal physiological functions of cells, the short application will have less chance to induce resistance and side effects. Current clinical trials of PTK inhibition in cancer therapies have tested the dose, route, safety, and efficacy of small chemical inhibitors for receptor tyrosine kinases in human patients. The properties of Src inhibitors, such as their basic chemical structure, IC_{50}, and selectivity, have also been documented through cancer-related studies (127) (Table 1). Among these chemicals, inhibitors from the pyrrolopyrimidine series have been well characterized. They are potent, reversible, and relatively selective and exhibit sufficient oral bioavailability in vivo (127). The invaluable information from cancer studies will accelerate our research of clinical application of these inhibitors in acute inflammatory responses.

Shall we use inhibitors to target individual Src PTK members? As discussed in this article, Src activation appears in multiple cell types; together, they constitute the excessive inflammatory responses. However, with the CVB3 infection-induced cardiomyopathy as an example, the importance of lymphocyte activation in this pathological process is clearly demonstrated (79). Targeting Lck specifically with PP1 or PP2, rather than SU-6656, may attenuate activation of lymphocytes and thus may have fewer side effects. The immediate innate responses to microbial pathogens are primarily mediated by leukocytes, such as neutrophils and macrophages. These cells can phagocytose and kill pathogens and produce a variety of inflammatory mediators and cytokines to coordinate additional host responses. Therefore, in the presence of infection, simply inhibiting acute inflammation could be detrimental. For example, specific neutralizing antibodies to chemokine receptor, CXCR2, reduced neutrophil infiltration in response to several strains of bacteria but also reduced clearance of these microorganisms and increased mortality (91, 97, 137). IL-10, an anti-inflammatory cytokine, blocks the expression of multiple proinflammatory cytokines and chemokines. Adenovirally mediated IL-10 gene delivery attenuated ischemia-reperfusion-induced lung injury in transplantation (36). Administration of exogenous IL-10 protected the lung from LPS- or immune-complex deposition-induced injury; however, it also impaired the direct killing of microorganisms (44, 123). Therefore, in the presence of infection, selectively blocking Src PTKs in tissue cells (such as endothelial cells to inhibit increased vascular permeability and epithelial cells to reduce tissue damage) but leaving the function of neutrophils, monocytes, and macrophages intact may potentially reduce tissue injury without jeopardizing the host defense. On the other hand, when infection is not the major concern, blocking of Src PTKs in leukocytes may rapidly control the excessive inflammatory responses. These concepts are not only clinically important but also scientifically interesting. Unfortunately, the selectivity of inhibitors within the Src family PTKs is still limited (Table 1). New chemicals or specific antisense or siRNA targeting different isoforms of Src PTKs need to be explored for this purpose.

As summarized in Table 2, most of pharmacological studies have used small rodents (mice and rats). For the purpose of clinical application, evidence from larger animals is required. Moreover, experiments with survival and organ functions as readouts should be considered. The relatively longer period of use of Src inhibitors will help elucidate the potential effects and side effects. Furthermore, the type of animal models should also be considered carefully. For example, LPS, especially LPS with high purity, has been commonly used as a model to study the mechanisms of inflammation and innate immune responses; however, animals usually respond to LPS differently than to bacteria and other naturally existing pathogens (128). Animal models that simulate clinical situations more closely should be tested before the consideration of clinical trials.

CONCLUSION

In summary, the role of Src family PTKs in acute inflammatory responses is a rising area of research. Because of the lack of specificity of Src inhibitors and redundancy of Src family members in their functions, the specific role of each Src family member in inflammatory responses is still largely unknown. However, application of small chemical inhibitors to effectively and specifically block Src PTKs could have a great clinical implication for diseases with acute inflammatory responses as underlying mechanisms. Cellular and molecular studies, together with studies using genetically modified animals, have demonstrated the complex role of Src PTKs in different cell types under different experimental conditions. The combined efforts between in vitro and in vivo studies, plus the combined forces between clinicians and basic scientists, will propel this research and, it is to be hoped, lead to much needed drugs for sepsis, ALI, ischemia-reperfusion injury (stroke and myocardial infarction), ARDS, and MODS.

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